

In vitro pull down and Co-IP assays

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 An abbreviated version of this protocol was published in eLIFE in Sep 2015

Geminivirus-encoded TrAP suppressor inhibits the histone methyltransferase SUVH4/KYP to counter host defense

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Detailed protocol

In vitro pull down assay

Binding/Washing Buffer

Final Concentration	Stock Concentration	250ml
* 50 mM Tris-HCl pH 7.5	1M	12.5ml
0.6 % Triton X-100	20%	7.5ml
0.2 % Glycerol	50%	1ml
150 mM NaCl	5M	7.5ml
** 0.5 mM 2-mercaptoethanol	14.3M	8.75ul
** 2 mM PMSF	33mM	1.7ml

* pH of the stock solution at room temperature. This is subject to change depending on the isoelectric point of your protein of interest. Try to keep a difference of at least one pH unit. Also, be aware that the pH of Tris buffers changes with temperature. This protocol is performed at 4°C, so the actual pH is ~0.5°C higher during the experiment.

** Add right before use.

Protocol

- In a 2 ml tube mix:
 - 2.5 ug prey protein times the number of samples (in my case, GST-fusion).
 - 50 ul (bed volume) of bait resin (in my case, amylose resin)
 - 1-2 ml of Binding buffer
- Pre-absorb the prey proteins by gently rotating 1h in 4°C
 - Prepare the tubes with the bait proteins ON ICE:
 - 1 ml Binding Buffer
 - 2.5 ug Bait protein (in my case, MBP-fusion)
- Clear the proteins by centrifugation for 2 minutes at 13,800 rcf in 4°C.
- Transfer 2.5 ug of prey protein to each bait protein tube. Specifically, divide the volume of clarified protein in the total number of samples and split evenly.
- Incubate rotating for 2 hours in room temperature.
 - Take aside the bait resin (in my case, amylose resin) that you will use for the experiment. I use 50 ul (bed volume) of bait resin per sample.
 - Equilibrate the bait resin with Binding Buffer. I do this by washing it with binding buffer 3 times. To collect the resin without damaging it, I centrifuge at < 600 rcf.
 - Make protein gels for:
 - Input control
 - Western blot for prey proteins
 - Coomassie staining for bait proteins
- Add the 50 ul (bed volume) bait resin, and continue incubation in the same conditions for 2 more hours in room temperature.
- Wash 3 times by adding 1ml of the binding buffer and rotating 10 minutes, collect the resin by centrifugation for 2 minutes at 1,500 rcf.
- Wash 3 more times with the binding buffer without the 10 minutes rotation intervals. The last time, take all the liquid out.
- Add 50 ul of 2X SDS and resuspend the beads. Boil 5 minutes at 95°C, follow by 2 minutes on ice, and 2 minutes of centrifugation at > 13,000 rcf in 4°C.

10. Load 10ul in gels.

Immunoprecipitation from *Nicotiana benthamiana* agro-infiltrated leaves

Buffers

IP Buffer	Stock	12.5 ml	50 ml
* 40 mM Tris-HCl pH 7.5	1 M	500 ml	2 ml
300 mM NaCl	5 M	750 ul	3 ml
5 mM MgCl ₂	1 M	62.5 ul	250 ul
2 mM EDTA pH 8.0	0.5 M	50 ul	200 ul
** 4 mM DTT	1 M	50 ul	200 ul
0.5 % Triton X-100	20 %	312.5 ul	1.25 ml
** 2 x Roche Complete EDTA-free protease inhibitor (1 pellet / 25 ml)	1 pellet/1 ml H ₂ O or 1 pellet	500 ul	2 pellets
** 1 mM PMSF	300 mM	42 ul	167 ul
5 % Glycerol	50%	1.25 ml	5 ml

* pH of the stock solution at room temperature. This is subject to change depending on the isoelectric point of your protein of interest. Try to keep a difference of at least one pH unit. Also, be aware that the pH of Tris buffers changes with temperature. This protocol is performed at 4°C, so the actual pH is ~ 0.5°C higher during the experiment.

** Add right before use.

Elution Buffer	Stock	1.5 ml
0.5 mM EDTA pH 8.0	0.5 M	1.5 ul
200 mM NH ₄ OH	6 M	50 ul

Protocol

- Grind 0.4 g of each sample in liquid Nitrogen using mortar and pestle.
- Mix the powder with 1.2 ml IP buffer (3 ml buffer : 1 g sample).
- Thaw the samples by gentle continuous stirring in the mortar, do not get bubbles. This step shall be done on ice. Transfer the liquid to a 2 ml tube.
- Centrifuge 15 minutes at full speed in 4°C.
 - While the sample is clarified, prepare 1.5 ml centrifugation tubes by cooling them on ice.
 - Also, take the anti-Myc beads and keep them on ice.
- Take out the supernatant to the cold 1.5 centrifugation tubes and centrifuge again for 15 minutes at maximum speed (21,000 g) in 4°C.
 - Prepare The anti-Myc-Conjugated Agarose Beads:
 - Prepare 15 ul of beads per sample to be assayed.
 - Spin down for 3 minutes at 1,600 g in 4°C. Remove the supernatant.
 - Wash 3 times with the IP buffer; each time add ±1 ml of IP buffer and invert several times until the beads are completely in solution, then centrifuge 3 minute at 1,600g in 4°C. Remove the liquid.
 - The last time, remove the liquid and keep the beads on ice.
- Collect the input sample by putting 50 ul of the supernatant from step 5.a to a clean tube, then add 10 ul of 6X SDS buffer.
- Take the remaining supernatant (clarified solubilized protein extract) out to incubation with the anti-Myc conjugated beads from step 5.a.iv. Add the supernatant to the tubes with the equilibrated beads and incubate rotating for 2h in 4°C.
- After the incubation, take the tubes back and place them on ice for about 5 min to decant the agarose beads, then spin down at 1,600 g for 3min.
- Wash the beads 5 times as follows: add 1 ml of IP buffer, rotate in 4°C for 10 minutes, collect the beads by centrifugation for 2 minutes at 1,600 g in 4°C.
- Do a sixth wash without rotation
- Elute with 400 µL of the Elution buffer; rotate in room temperature for 20 min then centrifuge at 1,600g for 3 min and transfer the supernatant to a clean tube (basic elution samples); store at -80°C.
- Regeneration of anti-Myc-conjugated agarose beads:
 - After elution, immediately wash the anti-Myc agarose beads with the IP buffer for 3 times before storing the beads in 50% suspension with glycerol-TBS-NaN₃ (50 mM Tris HCl pH 7.5, 150 mM NaCl, 50% Glycerol, 0.02% NaN₃) buffer at 4°C. All the beads can be combined.

Evaluation

- Run the gels for western blot. For IP, probe with anti-Myc antibody; for CoIP, probe with the antibody that is pertinent to detect the co-immunoprecipitated protein.

1. Castillo-González, C. and Zhang, X. (2021). In vitro pull down and Co-IP assays. Bio-protocol Preprint. [bio-protocol.org/rep790](https://doi.org/10.21956/bio-protocol.d790).
2. Castillo-González, C., Liu, X., Huang, C., Zhao, C., Ma, Z., Hu, T., Sun, F., Zhou, Y., Zhou, X., Wang, X. and Zhang, X.(2015). Geminivirus-encoded TrAP suppressor inhibits the histone methyltransferase SUVH4/KYP to counter host defense. eLIFE. DOI: [10.7554/eLife.06671](https://doi.org/10.7554/eLife.06671)

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